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14. ABSTRACT Prostate carcinogenesis is closely linked to aberrant activation of Ras or Ras signaling pathways (<i>e.g.</i> , Raf-MEK, or PI ₃ K pathways). The incidence of activating PI ₃ K mutations in early and advanced prostate cancer, or loss of PTEN is very high. Increased expression of the Ras/Raf/MEK/ERK pathway has been associated with advanced prostate cancer, hormonal independence and a poor prognosis. We have demonstrated that, when aberrantly activated, Ras is lethal to the cell unless a survival pathway also initiated by Ras is active. This survival pathway <u>requires</u> PKC δ . Unlike the classical PKC isozymes, PKC δ is not required for cell survival, and its inhibition or down-regulation in normal cells and tissues has no significant adverse effects. Inhibition of PKC δ in human and murine cells containing an activated Ras protein, however, initiates rapid and profound apoptosis. In this work, we are testing the <i>hypothesis</i> that inhibition or down-regulation of PKC δ in human and murine models of prostate cancer with aberrant activation of Ras signaling will cause targeted cytotoxicity in these tumors.					
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Table of Contents

	<u>Page</u>
Introduction.....	3
Body.....	4
Key Research Accomplishments.....	30
Reportable Outcomes.....	30
Conclusion.....	30
References.....	31
Appendices.....	32/ N/A

INTRODUCTION:

Although activating point mutations of Ras in prostate cancer, are not common, prostate carcinogenesis, in particular, is closely linked to aberrant activation of Ras or Ras signaling pathways (e.g., Raf-MEK, or PI₃K pathways). The incidence of activating PI₃K mutations in early and advanced prostate cancer, or loss of PTEN is very high. Increased expression of the Ras/Raf/MEK/ERK pathway has been associated with advanced prostate cancer, hormonal independence and a poor prognosis. Strategies have been devised to target various stages of Ras signaling, ranging from inhibiting protein expression *via* antisense oligonucleotides, to blocking post-translational modification with farnesyltransferase inhibitors, to inhibiting downstream effectors. Unfortunately, these have shown minimal if any activity in prostate carcinoma in clinical trials, or have been limited by toxicity. Because wild-type Ras and its downstream effectors are required for many critical cellular functions in normal cells, the therapeutic window for inhibiting Ras directly may be too narrow to exploit. Our novel alternative strategy would circumvent this limitation. We have demonstrated that, when aberrantly activated, Ras is lethal to the cell unless a survival pathway also initiated by Ras is active. This survival pathway requires PKC δ .¹⁻³ Unlike the classical PKC isozymes, PKC δ is not required for cell survival, and its inhibition or down-regulation in normal cells and tissues has no significant adverse effects. Inhibition of PKC δ in human and murine cells containing an activated Ras protein, however, initiates rapid and profound apoptosis. This molecular approach, targeting tumor cells containing a mutated oncogenic protein (and sparing normal cells), by altering a second protein or its activity, is sometimes termed “synthetic lethality.”^{4,5} Analogously, the dependency of tumor cells upon the activity of a non-oncogenic protein is sometimes termed “non-oncogene addiction.” *Hypothesis:* inhibition or down-regulation of PKC δ in human and murine models of prostate cancer with aberrant activation of Ras signaling will cause targeted cytotoxicity in these tumors. The Specific Aims of this Idea Proposal are: i.) Test the hypothesis that inhibition or down-regulation of PKC δ in human prostate cancer cell lines with dysregulation of Ras pathways selectively induces apoptosis. Using molecular modeling, multiple analogs of the current lead PKC δ inhibitor have been predicted to have more specificity and higher potency than the current lead compound. A collaboration with a leading medicinal chemist produced one analog with superior pharmacokinetics. Thirty six next generation other analogs have been synthesized and tested for activity and isozyme specificity *in vitro* and in tissue culture. The best one or two analogs will then be tested *in vivo* (below) in a head-to-head comparison with the current lead compound to identify an optimal PKC δ inhibitor. ii.) Determine whether constitutive activation of *selected Ras effector pathways alone* (PI₃K Pathway, *via* the commonly-occurring loss of PTEN or activating mutations in PIK3CA [p110 α]; or constitutive, aberrant activation of the MEK-ERK signaling pathway) is sufficient to make prostate cancer cells susceptible to apoptosis after PKC δ inhibition. iii.) Test the ability of PKC δ inhibitors to induce selective cytotoxicity in human *prostate cancer stem cells*. iv.) Test this targeted approach in *in vivo* models of human prostate carcinoma. A xenograft model will be employed, utilizing an activating Ras-mutant human prostate carcinoma cell line and a human prostate carcinoma cell line with aberrantly-activated Raf-signaling.

Innovation: Ras signaling is an attractive target for therapy of prostate cancer, but approaches aimed at Ras itself, or its critical signaling pathways, which are required in normal tissues, have had limited success. This “non-oncogene addiction” approach, however, exploits a weakness of

tumor cells with aberrant activation of Ras or Ras effectors – their absolute requirement for a survival pathway mediated by PKC- δ . In contrast, normal cells and tissues do not require PKC- δ . **Impact:** Current therapies for prostate cancer are inadequate, and aberrant activation of Ras or Ras pathways are common. A novel therapeutic modality selectively targeting prostate cancers with activation of Ras or Ras pathways would make a significant impact on the way prostate cancer is treated.

BODY:

TASK 1: Testing human prostate cancer cell lines for sensitivity to PKC δ inhibition

Status: IN PROGRESS

Methods: Assess the sensitivity of human prostate cancer cells with known activating mutations in H-Ras and wild-type Ras alleles to (non-Ras-mutated) prostate epithelial cells

Task 1a) Using siRNA to suppress PKC δ

Task 1b) Using new, specific small-molecule PKC δ inhibitors.

- Verify their PKC δ inhibitory activity and isozyme-specificity will be verified *in vitro* using purified PKC isozymes
- Testing their ability to induce apoptosis in prostate cancer cell lines, and selection of the most potent and PKC δ isozyme-selective for *in vivo* testing.

Assays: MTS assay for enumeration of cells at 48 and 72 hrs after treatment. LDH release assays or flow cytometry assays to assess cytotoxicity

Results:

Task 1a: siRNA – To demonstrate the specificity of this targeted approach, we first used PKC δ -specific lenti-viral based shRNA to efficiently knockdown PKC δ protein in a human prostate cancer cell line, DU145. We developed lentiviruses containing shRNA directed against PKC δ , or a scrambled shRNA. Viruses were titrated to determine moi for use. They were then used to infect prostate cancer cell lines. Even within 24 h, we observe very significant cytotoxicity, as assessed by LDH release. Parallel studies using these lentiviral vectors in other cell lines, including normal prostate cancer cells, were performed to validate PKC δ as a target for prostate tumor cells (these findings were presented in prior progress report).

Task 1b. We have describe the development of new specific PKC δ inhibitory molecules, and then show the results of the testing of these compounds on prostate cancer cell lines.

Pharmacophore Modeling and Development of new PKC δ Inhibitors: Highly isotype-specific PKC δ -inhibitory small molecules had not been identified by others to date. With our discovery and genetic validation that PKC δ is the specific target molecule for this Ras-targeted approach, we generated a pharmacophore model based on molecular interactions with “novel” class PKC isozymes. We established a initial pharmacophore model for PKC δ inhibitors, using mallotoxin/rottlerin [Lead Compound 1 (**LC-1**)] as a prototype structure for a moderately PKC δ -

specific inhibitor ($IC_{50}=5\mu M$), and incorporated protein structural data for PKC ϵ , another member of the “novel” group of PKC enzymes, which is also inhibited by mallotoxin. LC-1 is a naturally-occurring product, with moderate aqueous solubility, and oral bioavailability.⁴ It inhibits purified PKC δ at an IC_{50} of 3-5 μM *in vitro*, and inhibits PKC δ in cultured cells with an IC_{50} of 5 μM *in vivo* (but at 0.5 μM with exposure for >24 hrs, because of down-regulation of the PKC δ protein⁵). It is relatively selective for PKC δ over PKC α (PKC δ IC_{50} :PKC α IC_{50} is approximately 30:1). Furthermore, as we have published, this compound not only directly inhibits purified PKC δ , but also, over longer periods of exposure, significantly down-regulates PKC δ protein specifically, while having no effect on the levels of other PKC isozymes.⁵ Thus, this compound inhibits PKC δ at two levels. We have demonstrated “Ras-specific” activity of this compound in a number of publications and assays (see above). Daily *i.p.* doses of up to 40 mg/kg (800 μg /20 g) in mice do not produce any overt toxicity in our xenograft studies or others.⁴ Stability: Informal stability testing demonstrates >95% stability as a powder at room temp for >6 months. Toxicology: Pilot and published toxicity data indicate that the compound has a low toxicity profile (lowest lethal dose = 750 mg/kg, rat oral); 120 mg/kg (oral 6-day rat study) is the lowest toxic dose.^{6,7} This relative safety, combined with its *in vivo* efficacy, makes Lead Compound I attractive as a starting point for modification and drug development. We have demonstrated that better therapeutic candidates can be developed from it. The rationale for the development of new inhibitors was to improve the PKC δ -selectivity and potency. [Potential limitations on LC-1 itself as a therapeutic agent (despite its *in vivo* safety and activity) include its lack of high specificity for PKC δ ; its off-target effects, including inhibition of Cam Kinase III, MAPKAP-K2, and PRAK1 at IC_{50} s of <10 μM ; its non-PKC-mediated effects on mitochondrial uncoupling and modulation of death receptor pathways;^{8,9} and the lack of composition-of-matter IP around it, which would preclude eventual clinical development by big pharma.]

We designed and synthesized a 2nd generation set of analogs. In Analogs 1 and 2, the “head” group (A) was made to resemble that of staurosporine, a potent general PKC inhibitor and other bisindoyl maleimide kinase inhibitors, with domains B and C conserved to preserve isozyme specificity. Ease of synthesis was a major factor in the design of this head group. Analogs 3 to 5 have “head groups” from other known kinase inhibitors: 1) Analog 3 from the crystal structure of an inhibitor bound to CDK2 (pdb code: 1FVT); 2) Analog 4 based on purine, found in a number of different potent kinase inhibitors; and 3) Analog 5 from a potent inhibitor of aurora kinase (pdb code 2F4J). The first 2nd generation chimeric molecule, KAM1, was indeed active, and more PKC δ -specific (see **Table 2**, below), and showed activity against cancer cells with activation of Ras or Ras signaling.¹⁰ Another 2nd generation compound we generated (CGX, with a very different composition but which fit the pharmacophore model) has demonstrated activity against multiple human cancer cell lines with activated K- or H-Ras alleles *in vitro* and *in vivo* in animal models. On the basis of SAR analysis of KAM1, we have this year generated 36 new 3rd generation compounds.

The PKC δ inhibitory activity and isozyme-specificity of the 36 3rd generation analogs was assayed *in vitro*, using recombinant PKC isozymes, prior to comparative testing on prostate cancer cell lines.

Method: These assays utilize fluorogenic FRET detection (Z-lyte, R&D Systems) technology and peptide substrates, are robust and validated, and have been used to screen the 2nd and 3rd generation PKC δ inhibitors we have synthesized.

Results:

1. PKC δ Activity Assays of 3rd Generation Compounds

Recombinant PKC δ enzyme and FRET substrate. Compounds were tested at 5, 10 and 50 μ M. and results were shown in prior progress report. The selectivity of the inhibitors for PKC δ were assessed by comparison with PKC α -inhibitory activity, using recombinant PKC α enzyme and FRET substrate.

The information from the enzymatic activity/inhibitor assays above were compiled into a summary table (**Table 1**) for purposes of comparison.

Compound	PKC δ IC ₅₀	PKC α IC ₅₀	Cytotoxicity relative to Rottlerin			
			DU145	HRasG12V NP-PCSC	P-PCSC	P-PCSC
Rottlerin	4.4		xx	xxx	xxxx	xx
KAM-1	5.6					
B058	5.8		o	o	o	x
B071	3		o	o	o	o
B095	8		x	o	o	xx
B097	9.5		xx	o	o	o
B106	0.08	200	xxx	xxx	xxxx	
B108	3		o	o	o	o
B109	0.09	800	o	o	o	o
B111	2.5	100	x	o	o	
B112	6		xxx	xx	xx	xx
B117	4.5		o	o	o	o
B118	? 10		o	o	o	o
B121	4		o	o	o	o
B125	0.25	800	x	o	o	x
B128	6.5		xxx	o	o	xx
B129	6.5		x	o	o	x
B130	4		o	o	o	o
B131	10		o	o	o	o
B136	14		x	o	o	o
B137	3		o	o	o	o
B141	4.8		x	o	o	o
B142	3.5		o	o	o	o
B143	6		o	o	o	o
B146	5		o	o	o	o
B147	5.8		x	xx	o	x
B148	4		o	o	o	o
B149	0.31	>1000	x	xx	xx	o
B150	0.625	800	o	o	o	o
B151	< 0.05	>1000	o	o	o	o
B152	< 0.05	>1000	o	o	o	o
B153	3		o	o	o	o
B154	23		o	o	o	o
B155	3		o	o	o	o
B156	7.5		o	o	o	o
B157	5		o	o	o	o
B158	4.5		o	o	o	o
B159	7		xxxx	xx	xx	xx

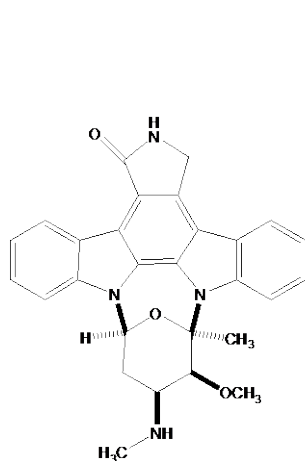
Table 1: Summary PKC δ and PKC α inhibitory activity of 36 3rd generation compounds, expressed as IC₅₀. Summary of relative cytotoxicity on multiple prostate cancer cell lines, relative to rottlerin.

Interpretation: Certain of the 3rd generation compounds show substantially greater PKC δ inhibitory activity and specificity than LC-1 or 2nd generation compounds. For example, one such novel compound (“B106”) is much more potent than LC-1 (**Table 1**), producing substantial cytotoxicity against Ras-mutant tumor lines at concentrations ~40 times lower than LC-1. This compound is also active *in vivo*, in a Ras-mutant cell xenograft assay. Both LC-1 and B106 dramatically inhibited clonogenic capacity of Ras-mutant tumor cell lines after as little as 12 h exposure. A newer derivative of this particular compound (CGD63), not yet optimized with respect to drug-like properties, has a PKC δ IC₅₀ in the range of 0.05 μ M (compared to 3 μ M for LC-1), is 1000-fold more inhibitory against PKC δ than PKC α *in vitro*, and produces cytotoxic activity against Ras-mutant cells at nM concentrations. (Specificity for PKC δ over classical PKC isozymes, like PKC α , is important: inhibition of PKC α is generally toxic to all cells, normal and malignant, and would make our agent non-“tumor-targeted.”) We are therefore seeking to maximize PKC δ -isozyme-specificity for the inhibitors to retain the tumor-targeted cytotoxic properties. We will eventually test selected inhibitors against an

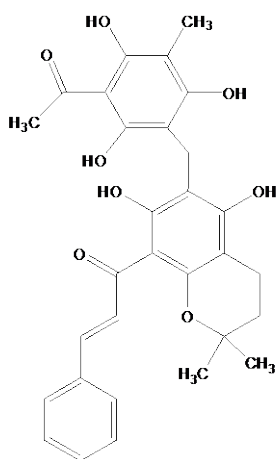
entire panel of recombinant PKC isozymes, including the classical, novel and atypical classes.

Table 2 compares the 3 generations of PKC δ -inhibitory compounds tested to date.

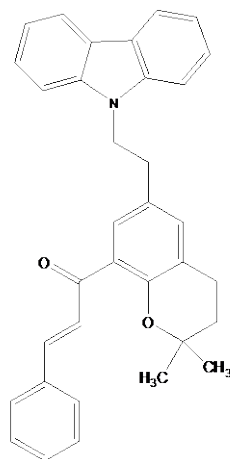
PKCδ inhibitors



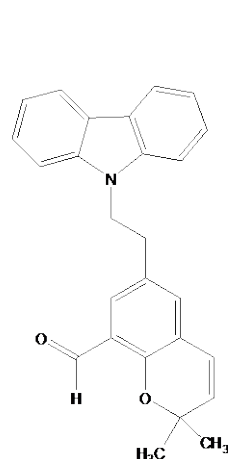
Staurosporine



Rottlerin



KAM1



BJE6-106

Generation	PKCδ IC ₅₀	PKCα IC ₅₀	PKCδ/PKCα Selectivity Ratio
1	3 μM	75 μM	28-fold
2	2 μM	157 μM	56-fold
3	0.05 μM	50 μM	1000-fold

Table 2

3. Testing of 3rd Generation PKCδ Inhibitor Compounds in Prostate Cancer Cell lines

Materials and Methods:

- Cells were grown on 60 mm tissue culture dishes, seeded to 1 x 10⁵ cells per well:
- Cells were allowed to grow 24 hrs at 37°C and 5% CO₂.
- On treatment day, media was removed from each plate and replaced with either vehicle or test compound in growth media
 - ♦ DMSO (vehicle for compounds)
 - ♦ Compounds tested at various concentrations
- At 48 or 72 hrs, cells were harvested, and viable cell mass quantitated via MTT or MTS assay.

We initially tested the entire panel of 36 3rd generation compounds against a prostate cancer cell line with an activating Ras mutation. The compounds were prepared in stock solutions. Results from representative cytotoxicity assays were shown in the prior progress report. We reported that certain 3rd generation compounds (106, 147, 149, 112 and 159) showed toxicity against this cell line comparable to LC-1 or greater than LC-1. Compound 106 ("B106") consistently showed the

most consistent and highest activity and was chosen as the lead compound for the subsequent studies.

B106 was tested at multiple concentrations against a panel of human prostate cancer cell lines with activation of Ras signaling pathways, and compared to LC1 (rottlerin) or vehicle.

Approach:

MTS Assay: DU145, LNCaP, PC-3, pZ-HPV-7 + 1, 2, 5, 10, 20 or 40uM Rottlerin x 96hrs. Treated on 3rd day after plated cells.

DU145, LNCaP, PC-3 (Prostate Cancer Cell Line), pZ-HPV-7 (Immortalized Prostate Cell Line) & Celprogen's Breast Cancer Stem Cells + 1, 2, 5, 10, 20 or 40uM Rottlerin x 24-96hrs.

Objective:

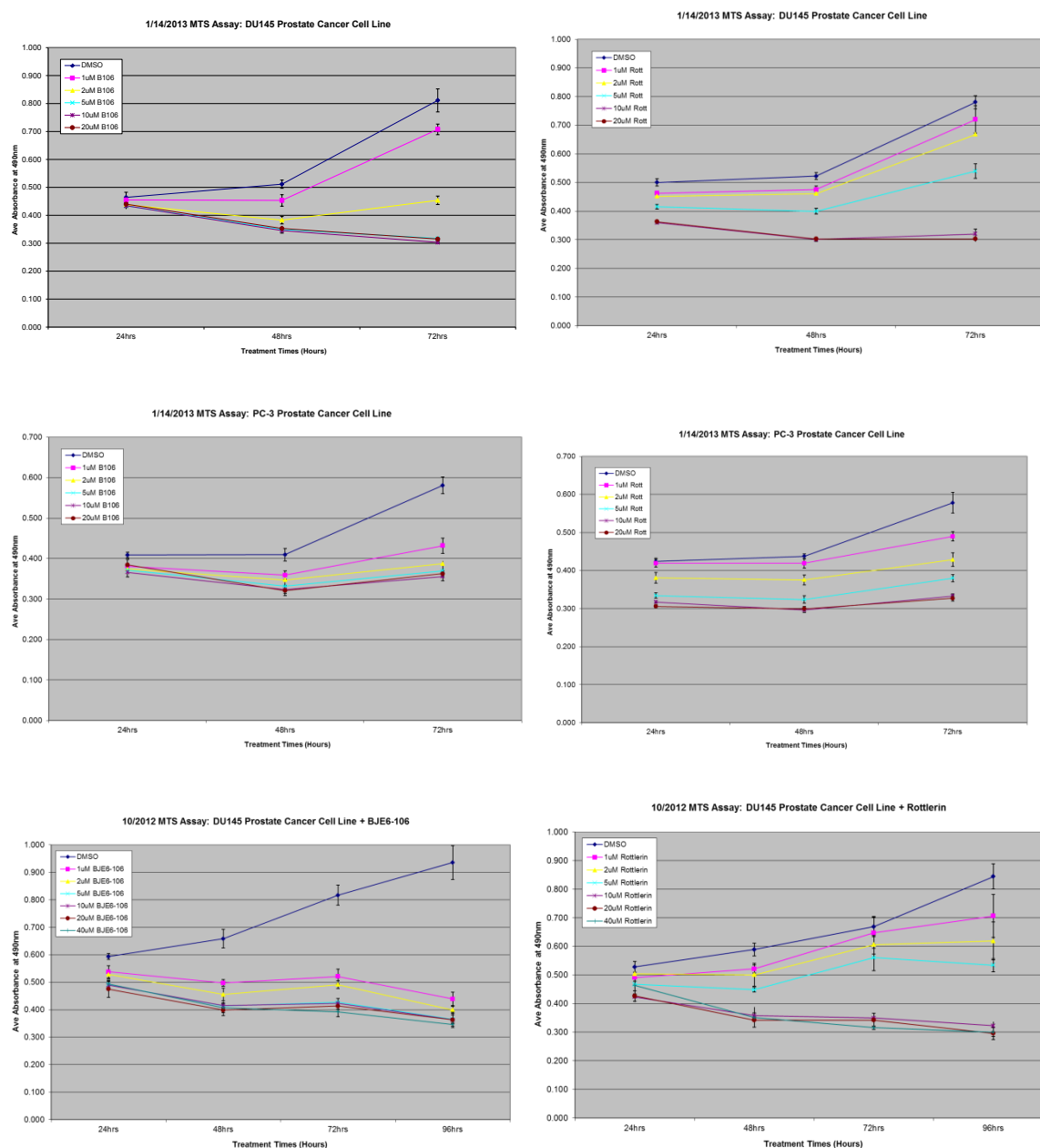
- To observe prostate cancer cell lines, immortalized prostate cells and Celprogen's Breast Cancer Stem Cells treated with 1, 2, 5, 10, 20 or 40 uM Rottlerin, PKC inhibitor. DU145 prostate cancer cells were the positive control for the effects of Rottlerin.

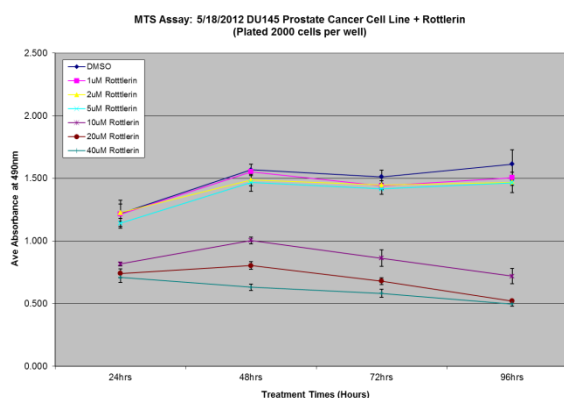
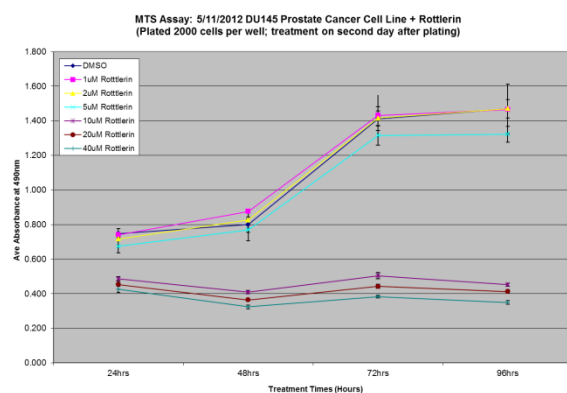
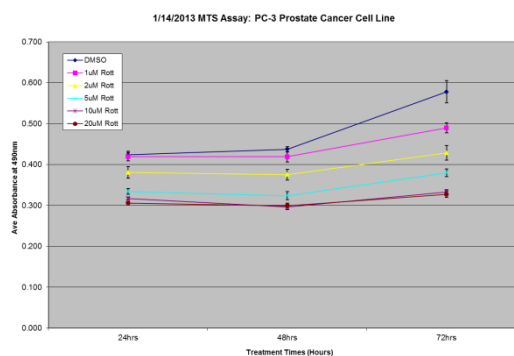
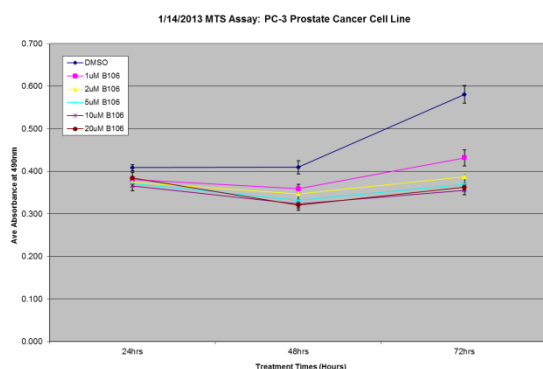
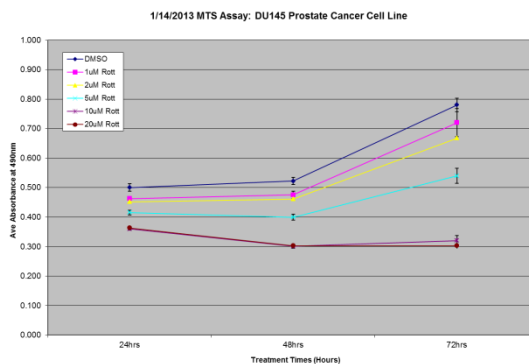
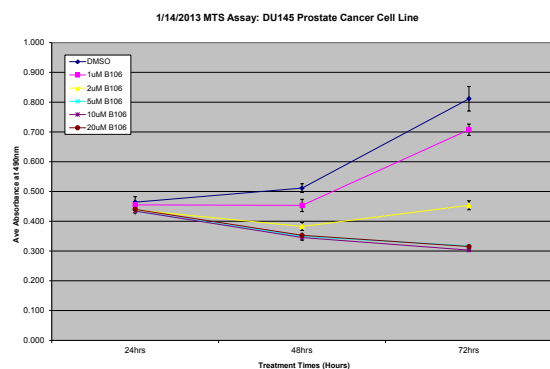
Materials and Methods

- Day 0: Cell plating day
 - Cells were plated at 2000 cells per well in 96 well plates. Quadruplicate samples were plated and grown at 37°C in 5% CO₂. Cells were allowed to grow for three days.
 - DU145 prostate cancer cells: 10%FBS (Invitrogen); Dulbecco's Modification of Earle's Media (MediaTech); 2mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; Passage 10.
 - LNCaP prostate cancer cells: 10% FBS (Invitrogen); RPMI 1640 (MediaTech); 2mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200 ug Streptomycin/ml (Invitrogen)); Passage 4.
 - PC-3 prostate cancer cells: 10% FBS (Invitrogen); Dulbecco's Modification of Earle's Media (MediaTech); 2 mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; Passage 10.
 - pZ-HPV-7 immortalized prostate cells: Keratinocyte Serum Free Media; 2 mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200 ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; with trypsin inhibitor use for trypsin neutralization; Passage 7.
 - Breast Cancer Stem Cells (Celprogen): Celprogen's media with serum for Breast Cancer Stem Cells; Passage 7. Plated on Celprogen's Breast Cancer Stem Cell extracellular matrix.
- Day 3: Treatment day
 - Media was removed from each well and replaced with 0.1ml of treatment prepared in fresh growth media and filter sterilized:
 - DMSO (Fisherbrand), vehicle.
 - Rottlerin (EMD Chemical), 40 mM stock in DMSO, aliquoted, not re-frozen.
- Day 4 (24 hr tmt), Day 5 (48 hr tmt), Day 6 (72 hr tmt), Day 7 (96 hr tmt):
 - Observations were made on the confluency of treated cells compared with vehicle treatment.

- MTS Assay was performed at each time point (CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega)) as described by manufacturer.
 - 20 ul of the assay buffer was added to each well. Cells were incubated for one hour at 37oC in 5% CO₂ humidified atmosphere.
 - The absorbance at 490 nm was read on the Molecular Devices, SpectraMax 190 plate reader.

Figure 1: Relative Sensitivity of PrCa cell lines to 1st and 3rd generation PKC δ inhibitors.





Interpretation:

The third generation PKC δ inhibitor B106 was consistently and substantially more potent at inducing cytotoxic/cytostatic effects on all the prostate cancer cell lines with activation of Ras signaling examined than the 1st generation inhibitor (rottlerin, LC-I). IC₅₀ for B106 was consistently < 1uM, whereas IC₅₀ for rottlerin ranged from 6-10 uM.

* The effects of the 3rd generation PKC δ inhibitors on non-tumor cells was examined. The pZ-HPV-7 cell line was derived from primary human prostate epithelial cells by transformation with human papilloma virus. While not tumorigenic, they do exhibit some properties of transformed cells.

Approach:

MTS Assay: pZ-HPV-7 cells (Immortalized Prostate Cell Line) were treated with Rottlerin or B106 at the indicated concentrations for 96 hrs. MTS assay was then carried to quantitate cell growth.

Results: The pZ-HPV-7 cells were sensitive to PKC δ inhibition to some extent (**Fig. 2**). Whether this was caused by the transformation with HPV is not clear.

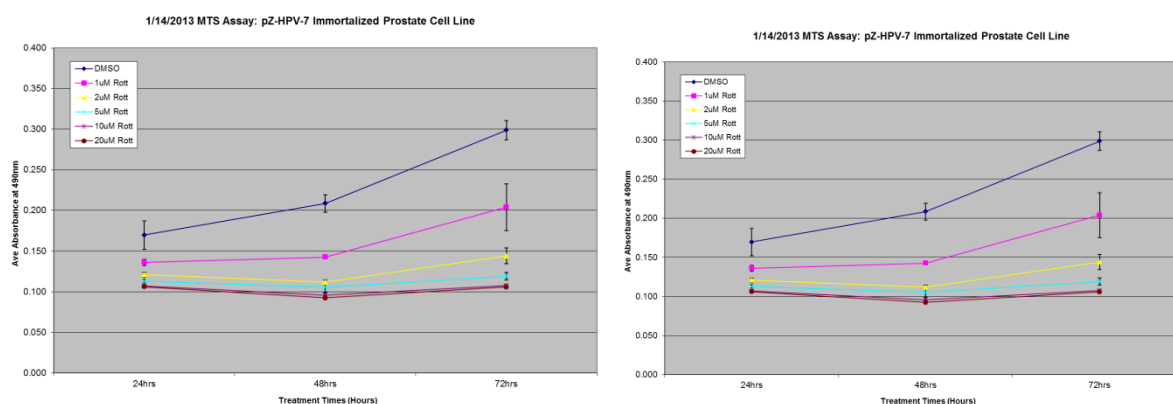


Figure 2

We decided therefore to examine the sensitivity of another cell line derived from a hormone responsive tissue, MCF 10A.

Approach:

MCF 10A cells were treated with Rottlerin or B106 at the indicated concentrations. MTS assay was then carried out at 24, 48 and 72 h to quantitate cell growth.

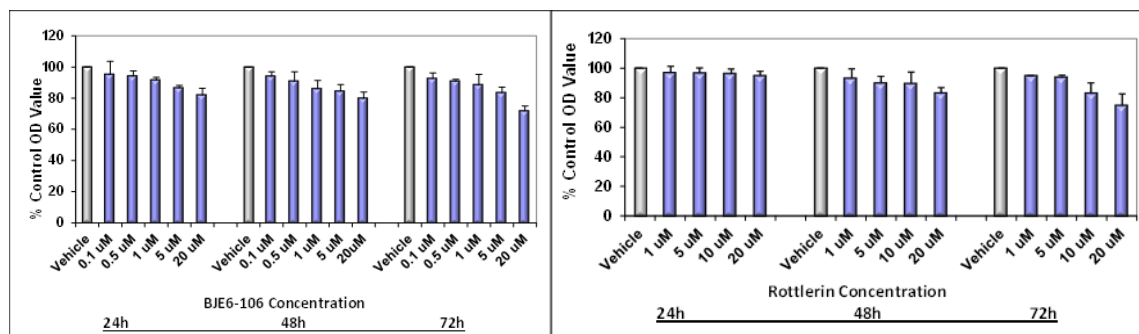


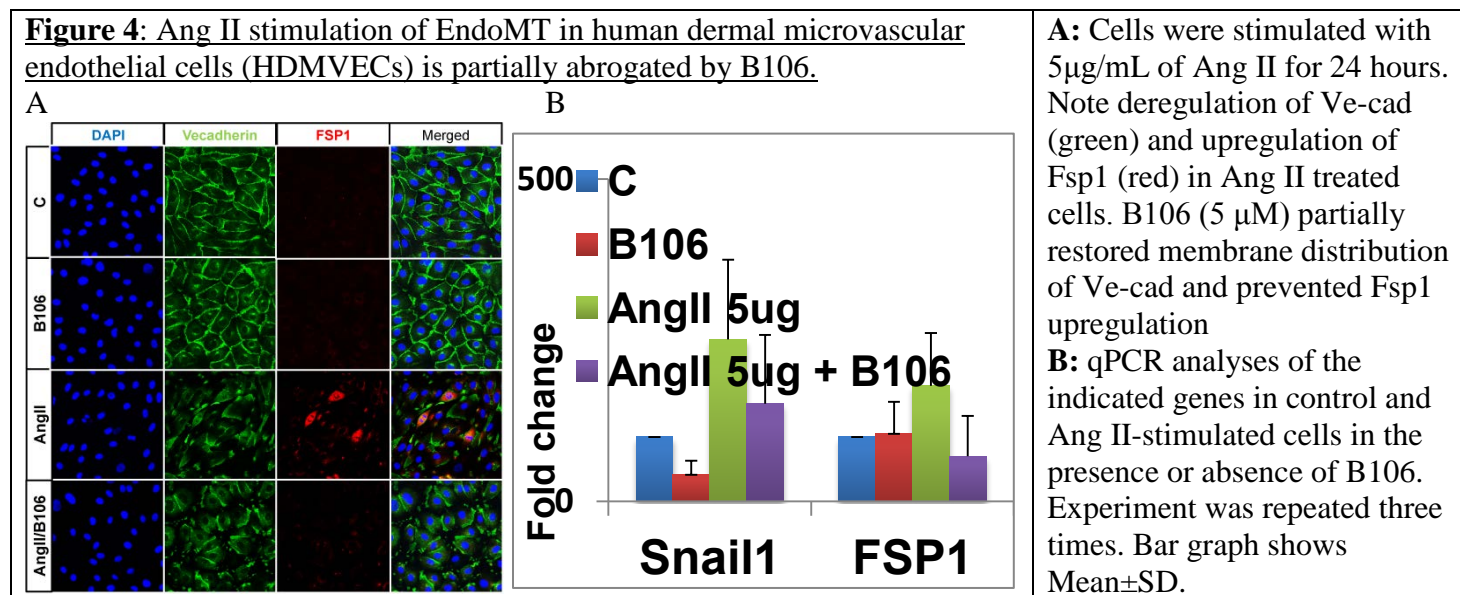
Figure 3

Results: The “normal” human epithelial cell line MCF 10A was insensitive to 1st and 3rd generation PKC δ inhibitors (**Fig. 3**). For MCF 10A, IC₅₀ for B106 was consistently >> 20 μ M, and IC₅₀ for rottlerin was also >> 20 μ M at all timepoints.

As an even more stringent assessment of the effects of 3rd gen PKC δ inhibitors on normal tissue, primary human microvascular endothelial cells were exposed to the compound in culture.

Approach:

In this particular experiment, primary human microvascular endothelial cells were exposed to vehicle, B106, angiotensin (and inducer of endo to mesenchymal transition), or angiotensin + B106. Effects on morphology and gene induction were analyzed.

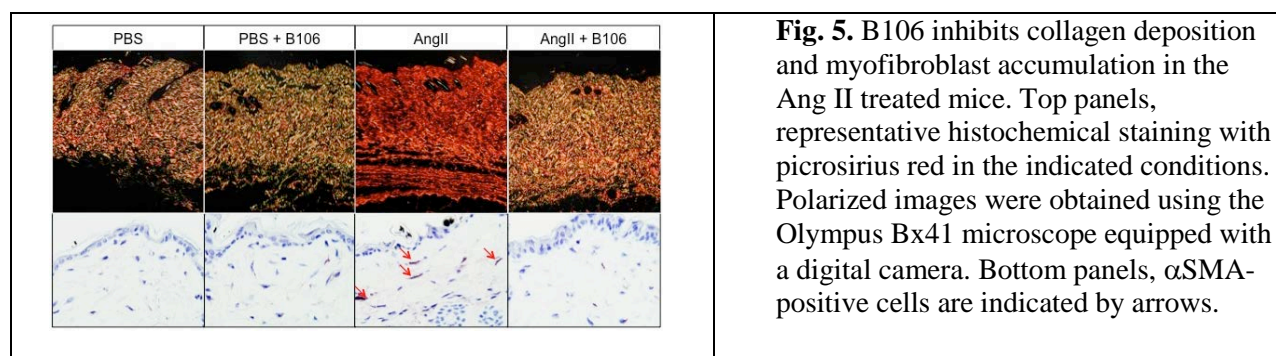


Results: Normal human endothelial cells were insensitive to this 3rd generation PKC δ inhibitor at a concentration of 5 μ M, as assessed by morphology (**Fig. 4A**) and induction of mesenchymal genes (**Fig. 4B**).

As the most stringent assessment of the effects of 3rd gen PKC δ inhibitors on normal tissue, B106 was infused into the dermis of mice over 4 days.

Approach:

In this particular experiment, B106 (5 μ M/day/mouse) was administered alone or together with the Ang II (an inducer of skin fibrosis) via the Alzet osmotic pump.



Results: Treatment with B106 alone had a minimal effect on skin collagen content as assessed by picrosirius red staining (**Fig. 5**). Collagen deposition was markedly increased in the skin of Ang II treated mice as illustrated by the yellow and red birefringence characteristic of the thicker, more densely packed collagen fibers and was visibly reduced by the addition of B106 with weaker greenish birefringence representing thinner, more loosely packed fibers 80. Treatment with Ang II + B106 showed reduced number of CD163+ macrophages (not shown) as well as myofibroblasts, when compared to Ang II treatment alone (**Fig. 5**).

Importantly, systemic administration of B106 in this model did not result in clinically apparent toxicity in these mice (as would be expected from the finding that PKC δ null mice grow and develop normally, and are fertile), indicating there is a therapeutic window for PKC δ inhibition.

Interpretation: B106, a potent and selective 3rd generation PKC δ inhibitor, is not toxic to normal cells either in culture or and *in vivo* at therapeutic concentrations.

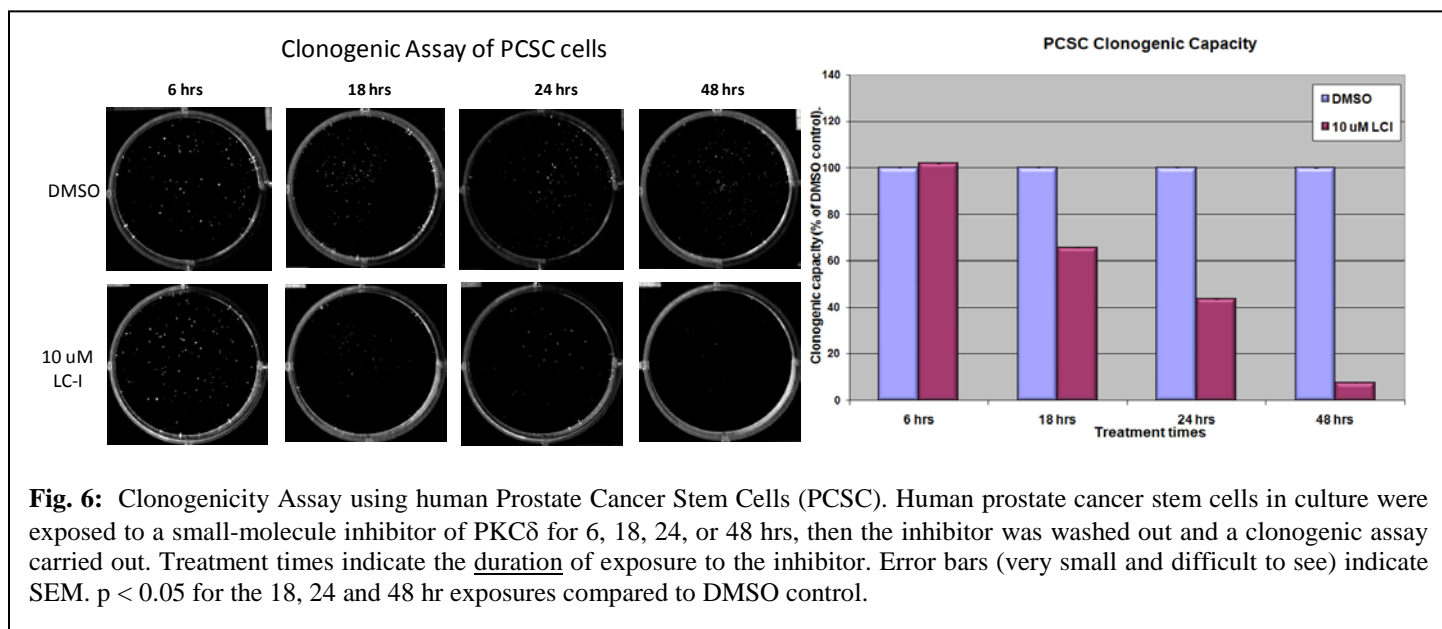
Task 1c) Determine the duration of PKC δ inhibition required to irreversibly initiate the apoptotic process.

Method/Assays:

1. Washout Studies: Exposure to inhibitors of PKC δ for different intervals of time, followed by washout, and assay of cell number over time. In this representative study, the DU145 cell line was used, and LC-1 (rottlerin) was used as the inhibitor (data shown in prior progress report).
2. Clonogenic Assays: Human prostate cancer stem cells in culture were exposed to a small-molecule inhibitor of PKC δ for 6, 18, 24, or 48 hrs, then the inhibitor was washed out and a clonogenic assay carried out. Colonies formed were enumerated. Treatment times indicate the duration of exposure to the inhibitor prior to replating (**Fig. 6**).

Interpretation: Cytotoxic effects on prostate cancer cells are observed after exposure to 1st generation PKC δ inhibitors for a period of 6 hrs. Longer periods of exposure produced progressively more toxicity. Replacing with fresh PKC δ inhibitors does not enhance the cytotoxic effect.

Follow-up Studies: These studies were repeated using a 3rd generation inhibitor, B106. Results confirmed that irreversible cytotoxic effects occur within 6 hr of exposure. The data is presented below in the section on Cancer Stem Cells (p. 24).



TASK 2: Determine whether constitutive activation of *selected Ras effector pathways alone* is sufficient to make human prostate cancer cells susceptible to apoptosis after PKC δ inhibition: (utilizing prostate cancer cells with aberrant activation of the PI₃K pathway or aberrant activation of the Ras-MEK-ERK pathway).

Status: NEARLY COMPLETE

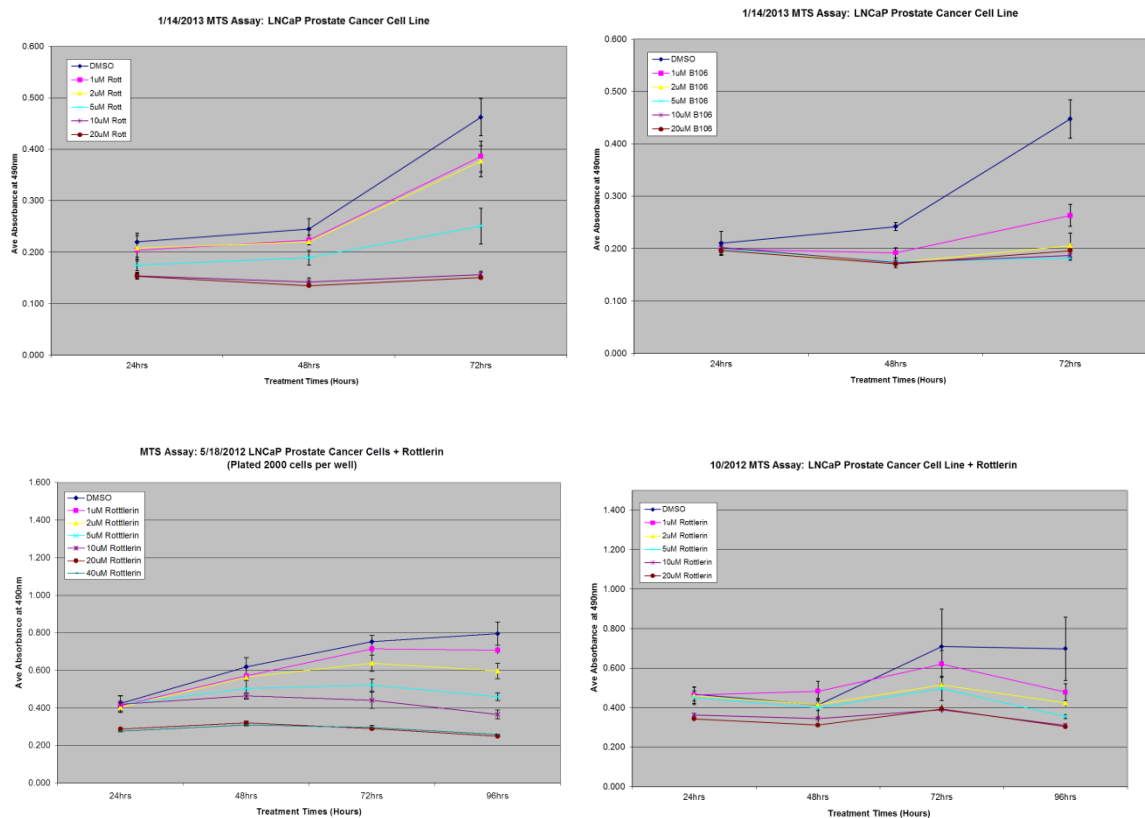
Task 2a) PI₃K Pathway: utilizing the LNCaP line with the commonly-occurring loss of PTEN [e.g., DU145]¹¹

Progress:

We have tested the most active 3rd generation inhibitor (B106) and our first generation compound (LC-I/rothlerin) against a prostate cancer cell line with activation of PI₃K pathway (LNCaP). Examples of these studies are shown below (**Figs. 7**).

Approach:

MTS Assay: LNCaP cells were treated with Rottlerin or B106 at the indicated concentrations for 96 hrs. MTS assay was then carried to quantitate cell growth.



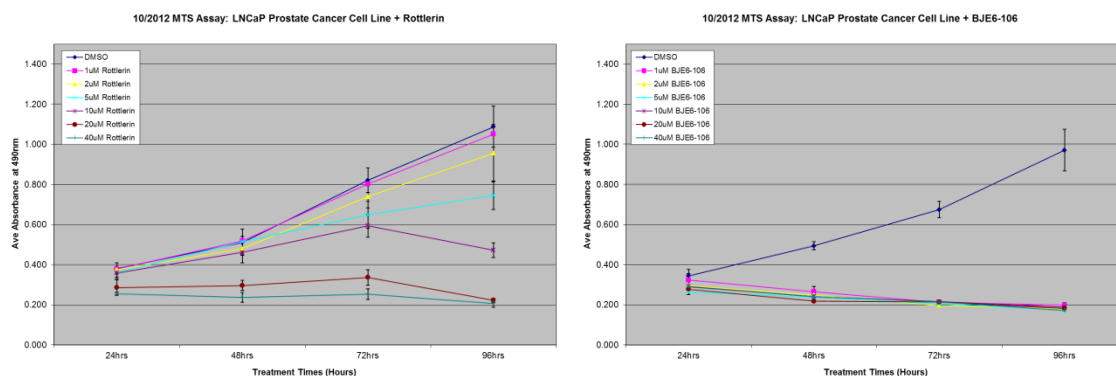


Figure 7

Results: IC_{50} for B106 was consistently $< 1\mu M$, whereas IC_{50} for rottlerin ranged from 6-10 μM (Fig. 7).

Interpretation: The third generation PKC δ inhibitor B106 was consistently and substantially more potent at inducing cytotoxic/cytostatic effects against this cell line greater than LC-1/rottlerin.

Task 2b) MEK-ERK Pathway: Human prostate cancer cell line CWR22Rv1 has constitutive, aberrant activation of the MEK-ERK signaling pathway, with wild type PTEN and PI $_3$ K signaling

Progress: The analysis of effects of PKC δ inhibitors on CWR22Rv1 has not yet begun, as we awaiting the development of 4th generation PKC δ inhibitors for this analysis. These lines will be tested for susceptibility to PKC δ knockdown by siRNA, or PKC δ inhibition by a small molecule inhibitor, with assay of cell numbers at 48 hrs by MTT assay.

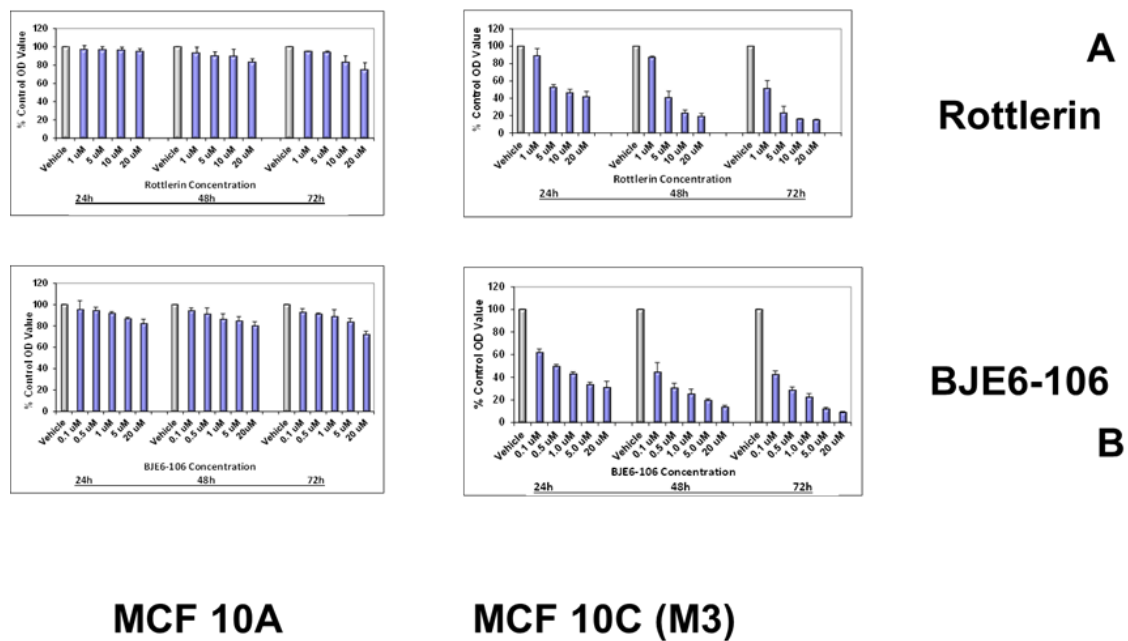
We have in the interim demonstrated that activation of the MEK-ERK pathway is sufficient to sensitize cells to PKC δ inhibitors. In these studies we transformed the “normal” human epithelial cell line MCF 10A to constitutively activate MEK-ERK (MCF 10C, or “M3”).

Methods: Cells were treated with vehicle or various concentrations of PKC δ inhibitors and MTS assays to assess cell number were done at 24, 48 and 72 hrs of exposure.

Results: MCF 10A cells are relatively insensitive to PKC δ inhibition, whereas the MCF 10C (M3) cells have become very susceptible (Fig. 8).

For MCF 10A, IC_{50} for B106 was consistently $\gg 20\mu M$, and IC_{50} for rottlerin was also $\gg 20\mu M$ at all timepoints. In contrast, for MCF 10C, at 72 hrs, IC_{50} for B106 was $\sim 0.1\mu M$, and IC_{50} for rottlerin was also $\sim 1.0\mu M$.

MCF 10A vs MCF 10C (M3)



Constitutive Activation of MEK/ERK sensitizes cells to PKC δ inhibition

Figure 8.

Interpretation: Constitutive activation of MEK/ERK is sufficient to renders cells sensitive to PKC δ inhibitors.

* Whether cancer cells need to be proliferating to become susceptible to PKC δ inhibition was studied in a system in which proliferation was slowed by serum deprivation.

Approach: Testing effects of growth rate on sensitivity to PKC δ inhibitors over the 96 hours of treatment. Cells were grown in 1%, 5% or 10% serum conditions, and exposed to rottlerin or vehicle. MTS assay was performed at 24, 49, 72 and 96 hrs.

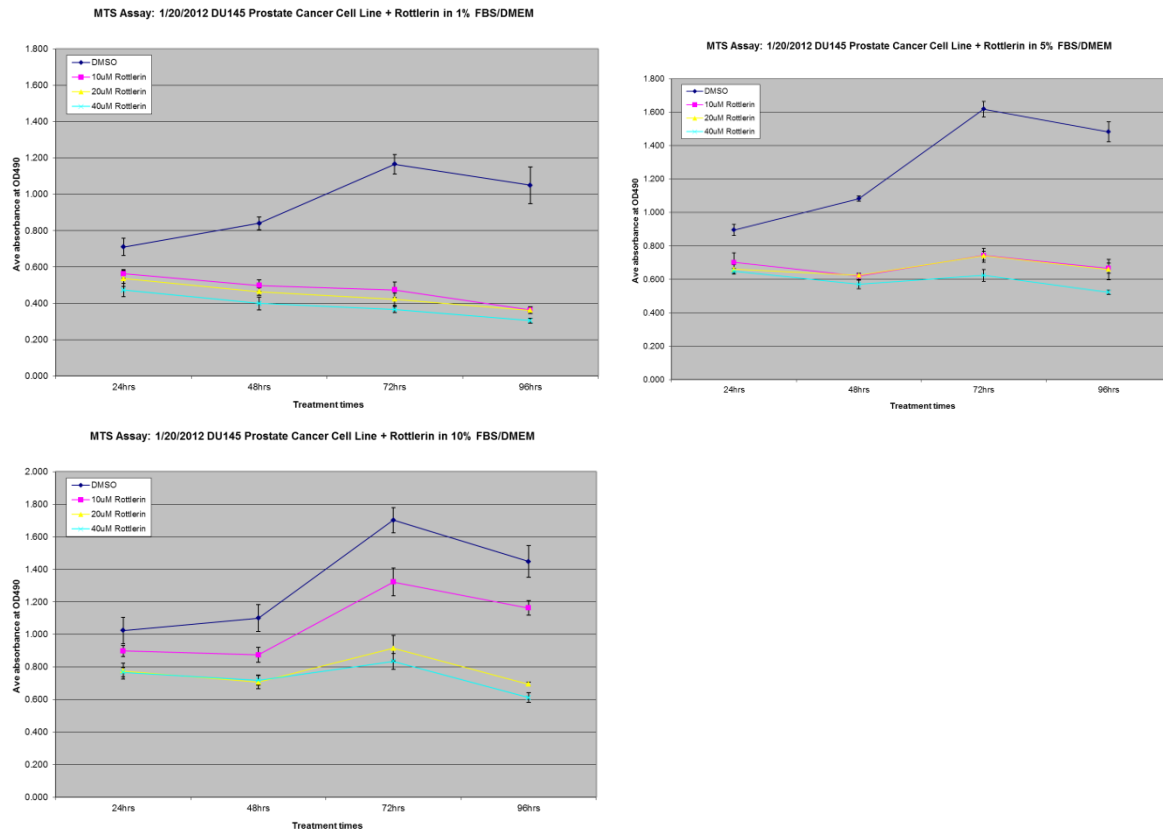


Fig 9. DU145 grown in reduced serum.

Results: Cell proliferation was slowed by PKC δ inhibitors even more efficiently when cells were proliferating slowly (1% or 5% serum), compared to normal culture growth conditions (10% serum) (**Fig. 9**).

Interpretation: Rate of proliferation does not alter susceptibility of human prostate tumor cells to PKC δ inhibitors.

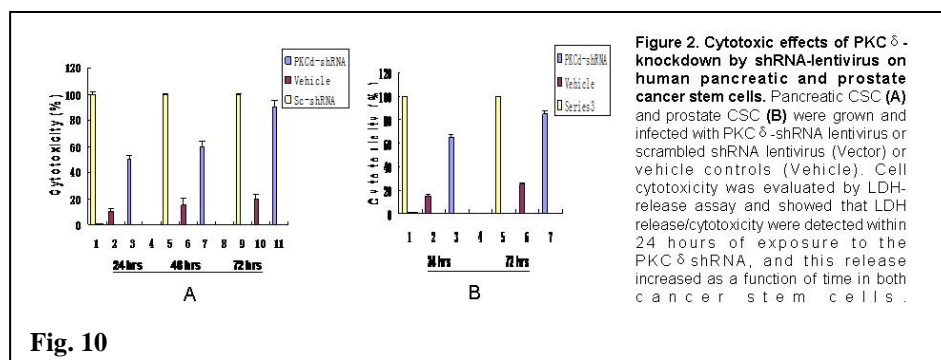
TASK 3: Test the ability of PKC δ inhibitors to induce selective cytotoxicity in human prostate cancer stem cells.

Human prostate cancer stem cells (Oct 4, telomerase, SSEA 3/4 , and AP positive), and normal breast stem cells, are purchased from Celprogen (San Pedro, CA), cultured under conditions which maintain their undifferentiated state, and tested for their susceptibility to PKC δ knockdown by siRNA, or PKC δ inhibition by a small molecule inhibitor, with assay of cell numbers at 48 hrs by MTT assay

Status: COMPLETED

Progress:

We first demonstrated that prostate cancer stem cells (CSC) are susceptible to PKC δ suppression using siRNA (shown in prior progress report) (**Fig. 10**).



We then tested LC-1 and 3rd generation PKC δ -inhibitory compounds on prostate cancer CSCs.

Methods: Primary human prostate cancer stem cells were treated with vehicle or various concentrations of PKC δ inhibitors and MTS assays to assess cell number were done at 24, 48 and 72 hrs of exposure. Examples of such studies are shown below (**Figs. 11**)

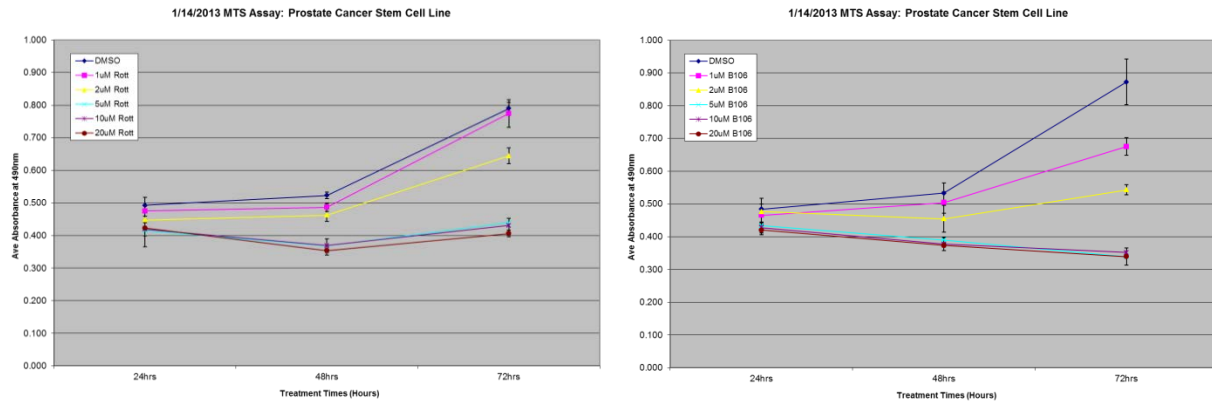
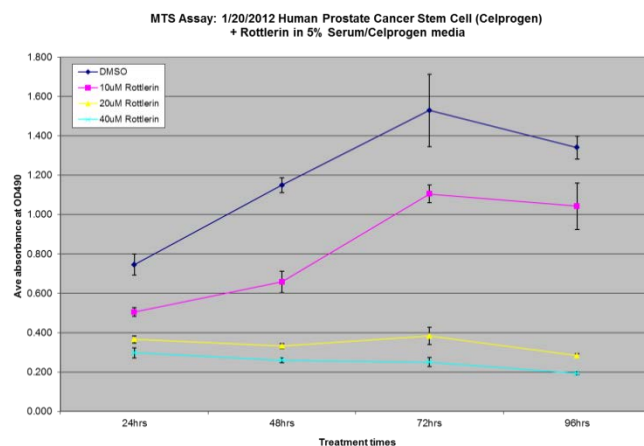


Fig. 11

Results: Primary human prostate cancer stem cells are sensitive to PKC δ inhibition, whereas the (Fig. 11). IC₅₀ for B106 was ~ 1 uM, and IC₅₀ for rottlerin was ~ 2.5 uM.

* Cancer Stem Cells are thought to be characterized by a slow proliferation rate, or dormancy in many tumors. Whether prostate cancer stem cells need to be proliferating to become susceptible to PKC δ inhibition was studied in a system in which proliferation was slowed by serum deprivation.

Approach: Testing effects of growth rate of prostate cancer stem cells on sensitivity to PKC δ inhibitors over the 96 hours of treatment. Cells were grown in 1%, or 5% serum conditions, and exposed to rottlerin or vehicle. MTS assay was performed at 24, 49, 72 and 96 hrs.



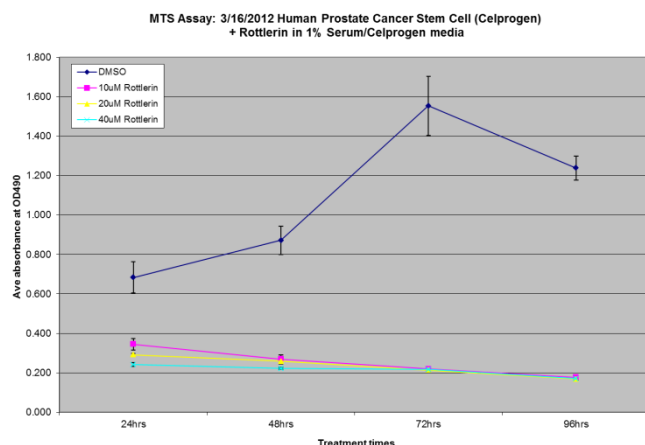


Fig 12. Human Prostate Cancer stem cells grown in reduced serum.

Results: Cytotoxicity was induced by PKC δ inhibitors efficiently when cells were proliferating slowly (1% or 5% serum), compared to normal culture growth conditions (10% serum) (**Fig. 12**).

Interpretation: Rate of proliferation does not alter susceptibility of human prostate cancer stem cells to PKC δ inhibitors

* CSC exhibit a spectrum of biological/functional, biochemical, and molecular features that are consistent with a stem-like phenotype, including growth as non-adherent spheres (clonogenic potential), superior (tumorigenic) ability to form a new tumor in an *in vivo* xenograft assays, unlimited self-renewal, and the capacity for multipotency and lineage-specific differentiation.⁶⁻¹⁰ In particular, CSCs are able to form colonies from a single cell more efficiently than their progeny¹¹ and to grow as spheres (tumor spheres) in non-adherent, serum-free culture conditions.¹² Sphere formation in non-adherent cultures has been used as a surrogate *in vitro* method for detecting CSCs from primary human tumors,¹³⁻¹⁷ and to purify a subpopulation of CSC-like cells from tumor cell cultures.

Our first objective was to determine if human prostate cancer cell lines can form tumorspheres.

Methods: Stem Cell Technologies Technical Bulletin, Tumorsphere Culture of Human Breast Cancer Cell Lines

1. Plated cell lines in complete MammoCult Media (Stem Cell Technologies) in 6well Ultra Low Attachment plates (Corning) at 20,000 cells/well.
 - a. DU145, 10%FBS/DMEM (Life Technologies/MediaTech) complete with Pen/Strep (MediaTech), L-Glutamine (MediaTech), HEPES (Fisher), passage 10.
 - b. LNCaP, 10%FBS/RPMI 1640 (Life Technologies/MediaTech) complete with Pen/Strep (MediaTech), L-Glutamine (MediaTech), HEPES (Fisher), passage 11.
 - c. PC-3, 10%FBS/DMEM (Life Technologies/MediaTech) complete with Pen/Strep (MediaTech), L-Glutamine (MediaTech), HEPES (Fisher), passage 7.
 - d. pZ-HPV-7, Keratinocyte-serum free media, passage 8.
 - e. Representative photos were taken at 3 days (**Fig. 13**) and 6 days (**Fig. 14**).

2. Quantitation method by transferring 6well TS and media to microcentrifuge tube; rapid spin down; aspirate media; gently resuspend TS with 1ml pipette; transferred to 96well non-sterile, assay plate; drew cross hairs under well to section quadrants for counting.

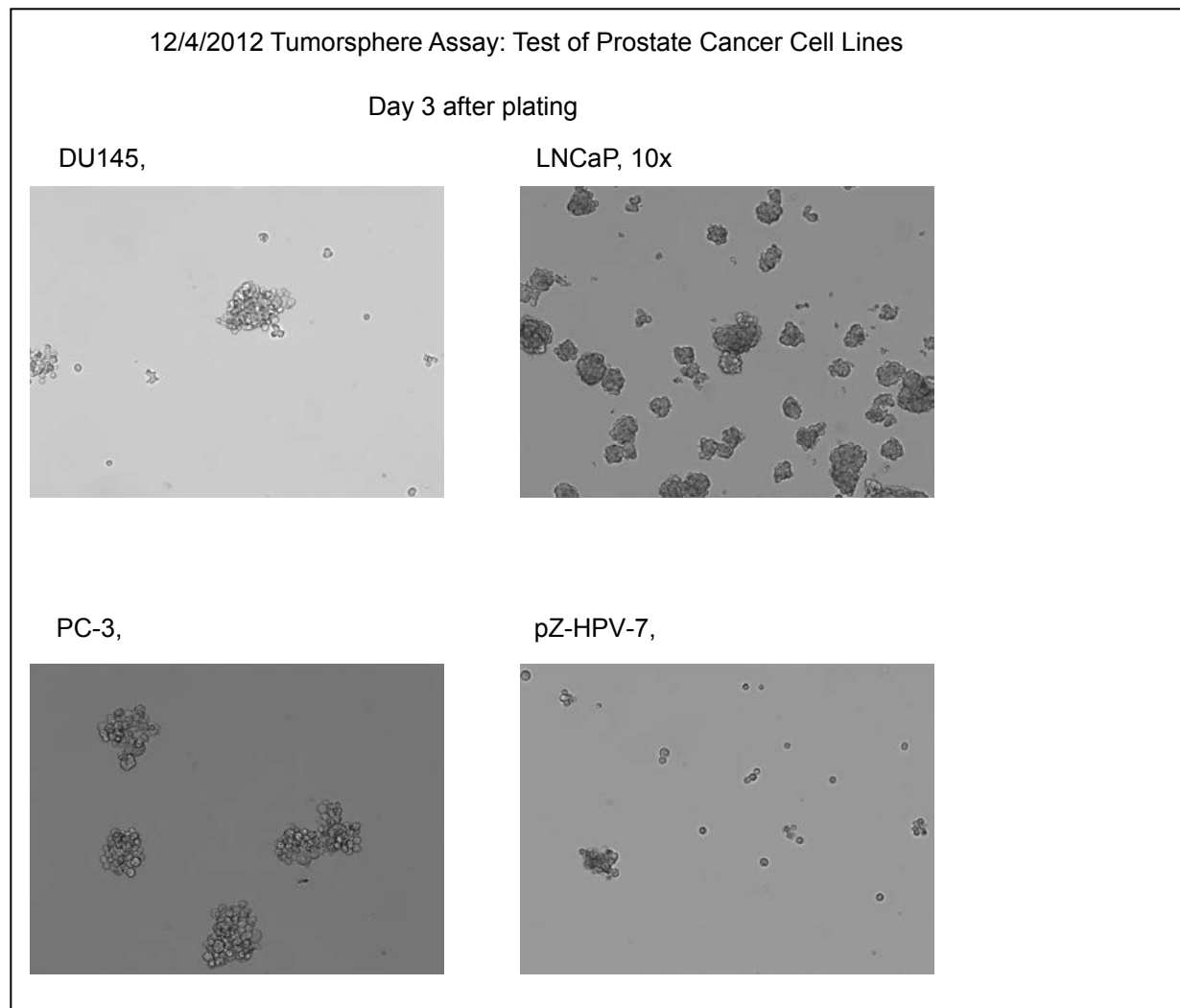
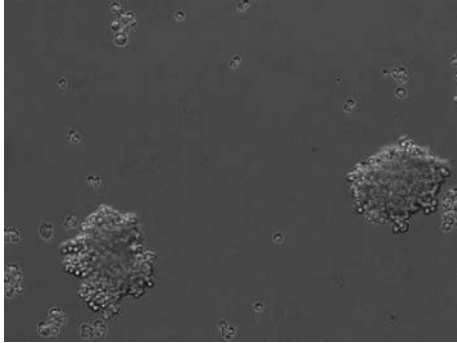


Fig. 13

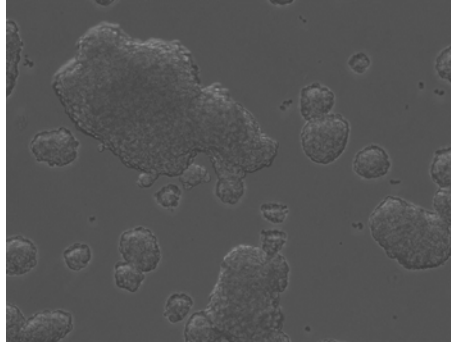
12/4/2012 Tumorsphere Assay: Test of Prostate Cancer Cell Lines

Day 6 after plating (12/10/2012)

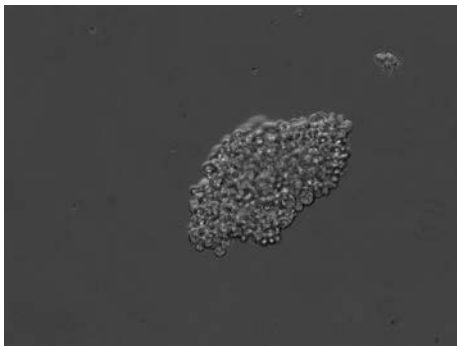
DU145, 10x



LNCaP, 10x



PC-3, 10x



pZ-HPV-7, 10x

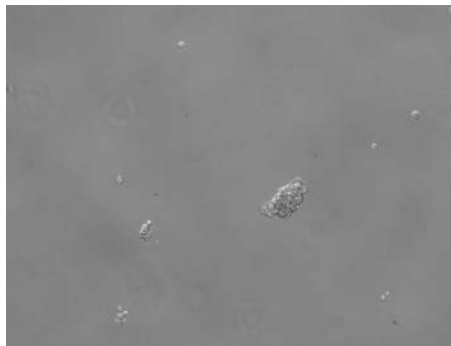


Fig. 14

Results: The human prostate tumor cell lines (DU-145, PC3, LNCaP) efficiently formed spheres at a frequency of about 2-5% of input cells (**Figs. 13-14**). The non-tumorigenic pZ-HPV-7 line did not.

We next determined whether 1st and second generation PKCδ inhibitors could inhibit the formation of tumor spheres by the CSC subpopulation.

Methods: Prostate cancer cells were plated and grown in the presence of rottlerin or B106 at the indicated concentrations, and sphere numbers quantitated.

Results: B106 at 1 and 5 uM, and Rottlerin at 10 uM were very efficient at inhibiting the formation of tumorspheres (**Fig. 15**).

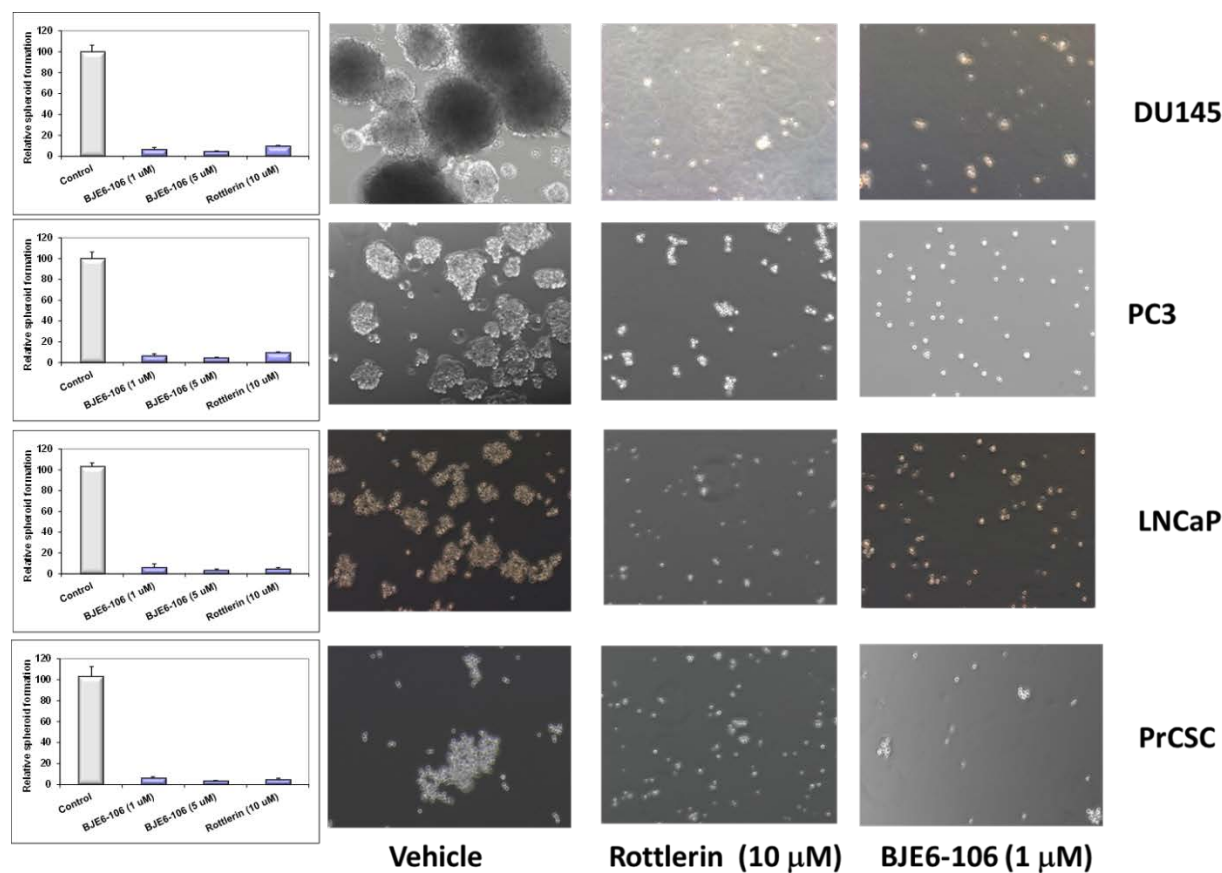


Fig. 15

Interpretation: PKC δ inhibitors efficiently inhibit the ability of prostate CSC from proliferating and forming tumors.

The phosphor-protein profiles of the tumorspheres compared with the “parental” tumor cells was examined to determine if the “tumorsphere” selection really selected out a subpopulation of CSC from the parental cells with different characteristics.

Methods: cell lysates were prepared and immunoblotted for ERK, pERK, JNK, pJNK, AKT, pAKT, and α -tubulin as a loading control (**Fig. 16**).

Results: The cells capable of forming spheres consistently had more pERK than the matched parental lines. pAKT levels were higher in the spheres than in the parental cells in the DU145 and LNCaP lines (**Fig. 16**).

Interpretation: The subpopulation with the ability to form spheres exhibit a different pattern of phosphoproteins, particularly pERK, than the respective parental lines. This hyperactivation of Ras pathway signaling may account for their susceptibility to PKC δ inhibition

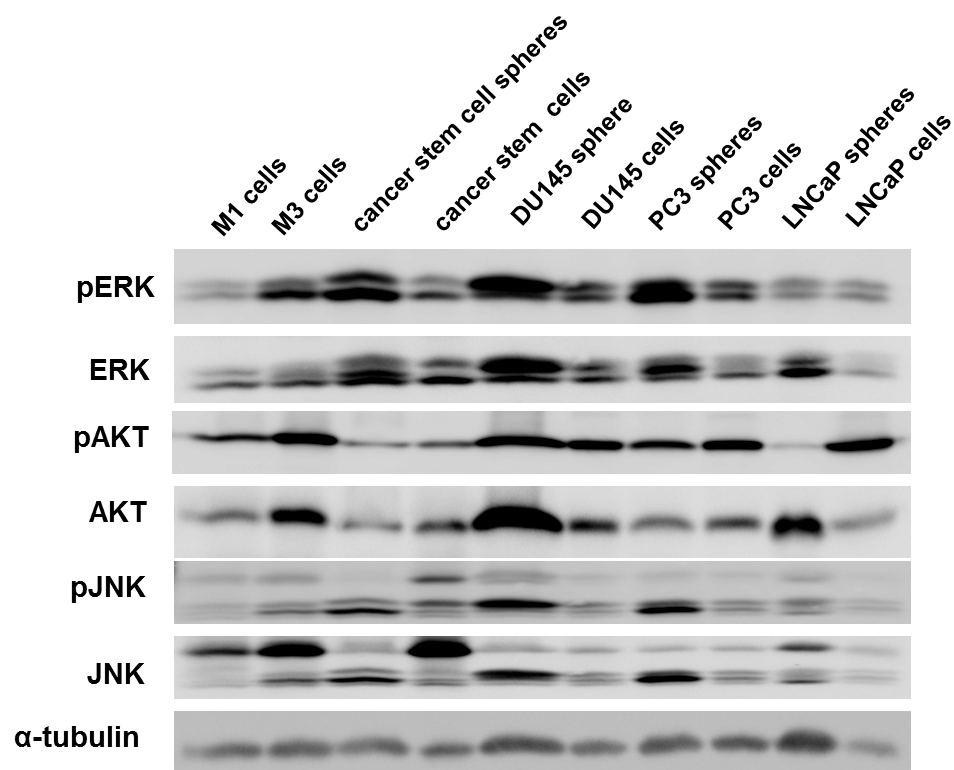


Fig. 16

TASK 4: Test this Ras-targeted approach in an *in vivo* model of human prostate carcinoma.

Progress: IN PROGRESS.

This task has been initiated. We initially established the MTD for B106, our lead compound at this time, then tested it against a xenograft model.

Methods: Test this targeted approach in *in vivo* models of human prostate carcinoma. A xenograft model has been employed, utilizing a human prostate carcinoma cell line with aberrantly-activated Ras-signaling (PC3). Two cohorts of 8 immunodeficient (nu/nu) mice each were implanted with a xenograft, one treated with vehicle control (100% DMSO) and one given the B106 PKC- δ inhibitor at the MTD, given i.p. in 100% DMSO. Tumor growth was serially quantitated.

Results: There was no statistically-significant effect of B106 on tumor growth compared to vehicle (DMSO) controls (**Fig. 17**).

Interpretation: This result was disappointing, given the high potency of B106 against prostate tumor cell lines in tissue culture. We believe at this time however that this result is due to poor bioavailability of the drug in this experiment. B106 is so hydrophobic that it must be given in 100% DMSO, and precipitates whenever any aqueous solvent is added, including ethanol. The drug appeared to precipitate when given i.p., and therefore little if any reached the circulation or tumor.

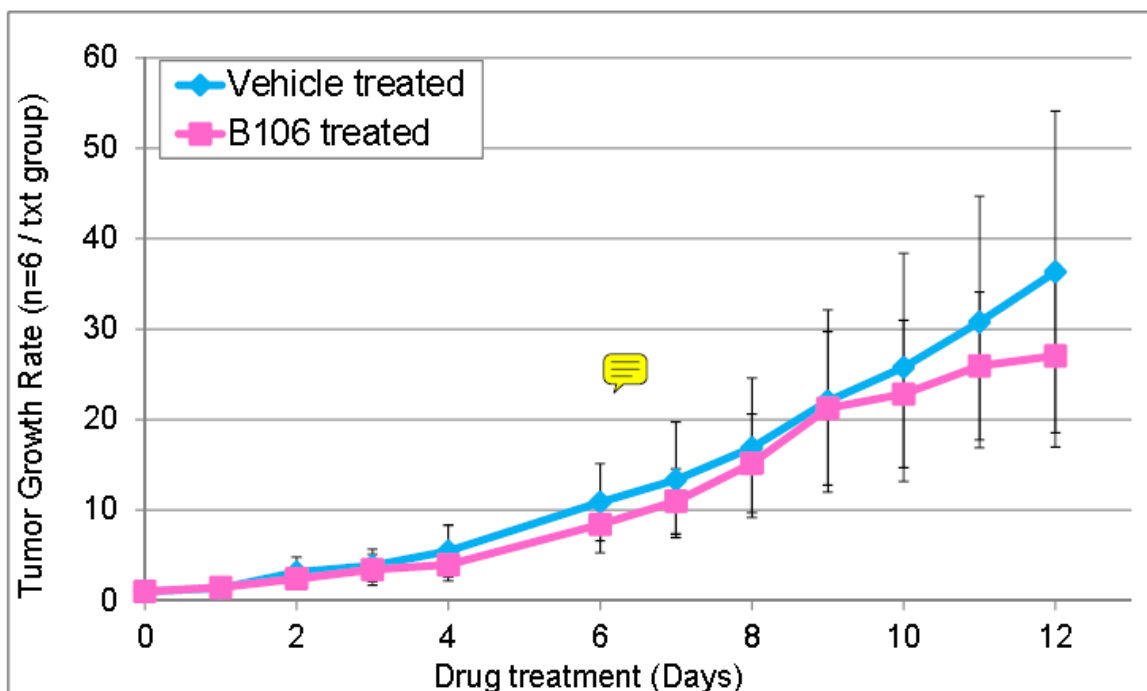


Fig. 17

We are therefore pursuing two different routes to improve the bioavailability of the drug: 1) using microparticle encapsidation techniques; 2) modifications to improve hydrophilicity.

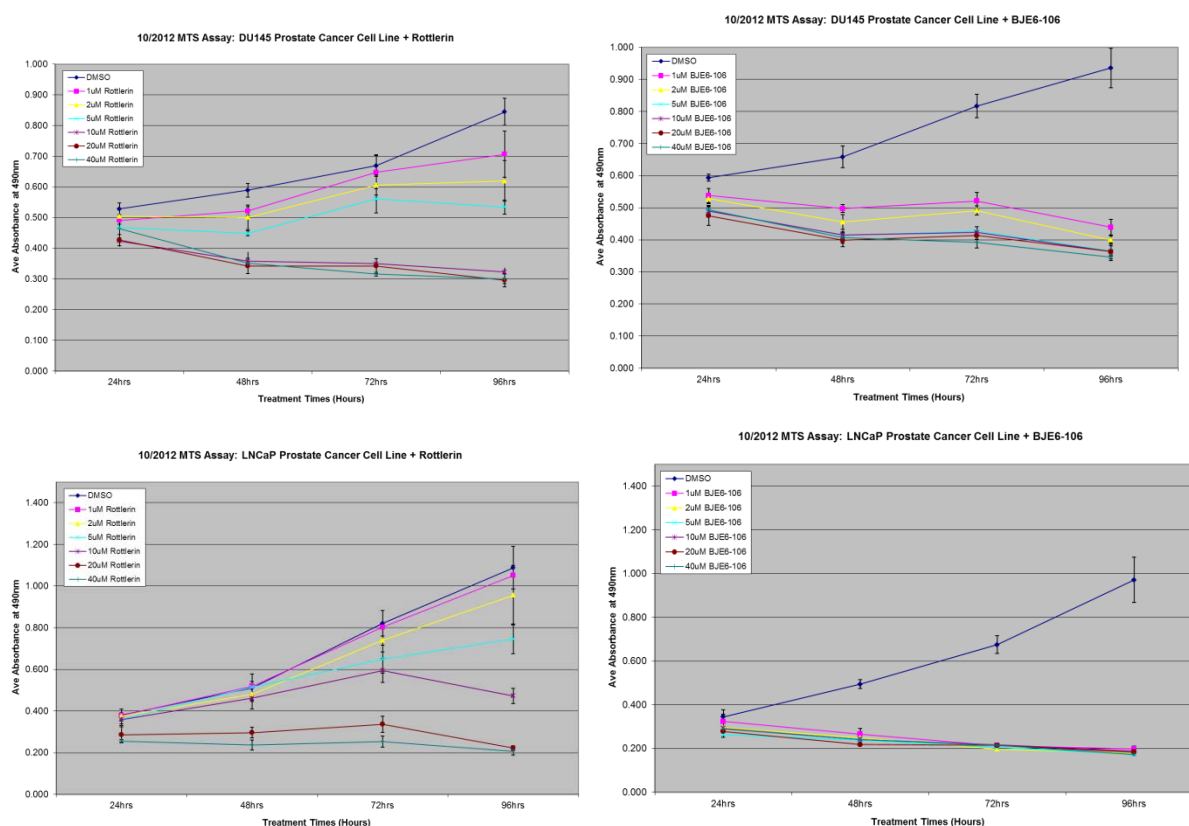
Microparticle Encapsidation:

Approach:

We have used proprietary liposome technology to bind the hydrophobic B106 into a lipid matrix.

MTS Assay: DU145 or LNCaP cells were treated with Rottlerin or liposomal B106 at the indicated concentrations for 96 hrs. MTS assay was then carried to quantitate cell growth.

Fig. 18. New formulation. MTT assay



Results: The LNCaP and DU145 cells were sensitive to rottlerin, as expected, and to the liposomal B106, with IC_{50} s in the range we found for the non-liposomal B106. IC_{50} for liposomal B106 was consistently < 1uM, whereas IC_{50} for rottlerin ranged from 6-10 uM (**Fig 18**).

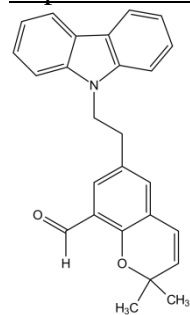
Interpretation:

Liposomal encapsulated B106 remains cytotoxic to prostate cancer cells. This type of “packaging” may be useful for *in vivo* delivery of the drug.

Chemical modifications of B106 to improve drug-like properties.

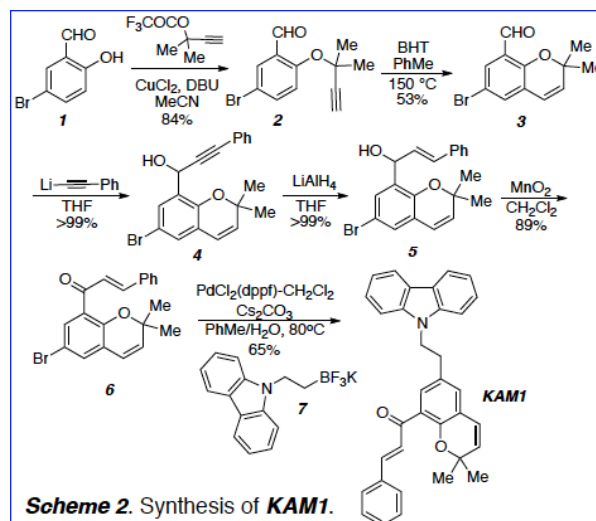
The second generation molecule showing activity against PKC δ (KAM1) was formed by combining structural elements of the broad spectrum protein kinase inhibitor staurosporine and rottlerin. The chromene portion of rottlerin was combined with the carbazole portion of staurosporine to produce KAM1. This was further modified to develop B106, which inhibits PKC δ with an IC₅₀ value of 50 nM and is 1000-fold selective versus PKC α . However, B106 displays limited solubility and preliminary data suggests rapid elimination, likely by metabolism. Therefore, modifications will be made to improve their solubility and metabolic stability. The proposed modifications are shown in **Figure 19**. These will be synthesized by our medicinal chemist collaborators using the synthetic approaches noted in **Figure 20**. We will start by simply adding polar groups to the B106 scaffold, which is thus far the most promising analog. Thus, as shown in Figure 19, R₁ and R₂, which are hydroxyl groups in rottlerin and are hydrogen atoms in B106, will be sequentially substituted with OH groups which should improve water solubility. In addition, we plan to perform an isosteric replacement of the aromatic CH groups (X and Z) with basic nitrogen atoms which will be protonated at physiological pH providing for additional water solubility and perhaps improved potency. Space does not permit a detailed description of the synthetic plan but it can be said that these new 4th generation analogs do not pose a significant synthetic challenge and are well within the expertise of our consult, Professor Robert Williams, and should be amenable to the basic synthetic chemistry platform that was developed to make KAM1 (Figure 20). Single substitutions will be evaluated at first, and then combinations of substitutions on the B106 core will be prepared. A total of 8-12 compounds will be synthesized and evaluated. They will be evaluated for solubility and octanol:water partitioning coefficient (logP). Further characterization of the pharmaceutical properties of these analogs will be carried out following evaluation of enzyme selectivity.

Figure 19. Proposed modifications to B106 to Explore Structure Activity Relationships and Improve Pharmaceutical Properties



R₁; R₂ = H or OH
X; Z = N or CH
R₃ = H, cinnamoyl, other R

Figure 20. Synthetic scheme used for KAM1



 <p data-bbox="253 570 326 598">B106</p> <p data-bbox="188 604 370 633">Modifications</p> <p data-bbox="483 570 602 598">Proposed</p>	
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The potency and selectivity of the new analogs will initially be evaluated for PKC δ and PKC α inhibitory activity using recombinant PKC α or PKC δ (Invitrogen) and the Omnia Kinase Assays (Invitrogen) with a ‘PKC kinase-specific’ peptide substrate. Incorporation of a chelation-enhanced fluorophore results in an increase in fluorescence (lex360/lem485) upon phosphorylation. The kits will be used according to the manufacturer’s instructions. For molecules that look promising (*e.g.*, low nM potency and >1000x selectivity vs PKC α , additional closely related kinases will be evaluated including PKC β I, PKC β II PKC γ , PKC ϵ , PKC η PKC θ , PKC ζ and PKC ι . The molecules with the optimal potency and selectivity will be further characterized. Criteria for advancement include at least 1000 fold selectivity versus PKC- α , which is important in many cellular processes and is a fundamental regulator of cardiac contractility and Ca²⁺ handling in myocytes, improved solubility of at least 10 ng/mL and octanol:water partitioning coefficient (logP) in the 1.5 – 3.5 range. Compounds that exhibit these characteristics will then be evaluated for their selectivity against other PKC family members. As B106 has already been shown to be safe when administered to mice at therapeutically active doses, this compound will be profiled against the other PKC family members (PKC- β I, PKC- β II PKC γ , PKC ϵ , PKC η PKC θ , PKC ζ and PKC ι). The profile of B106 will act as a template against which other compounds will be compared. Regardless of those results, compounds will be sought with at least 100-fold selectivity against the most biologically important other protein kinase C family members, including PKC γ (important in neuronal function) and PKC ϵ (important in apoptosis, cardioprotection from ischemia, heat shock response, as well as insulin exocytosis). Inhibitory activity against the other PKC family members will also be evaluated to fully profile the compounds.

Compounds with at least 1000 fold selectivity against PKC α , a logP of 1.5– 3.5 and solubility of at least 10 mg/mL will be further characterized for their biological activity. The ability of selected inhibitors to induce cytotoxicity in human prostate cancer cells will be assessed as we did for the 3rd generation compounds (Tasks I and II above). These compounds will also be evaluated for cytotoxicity on normal human endothelial cells (human umbilical vein endothelial cells, available from ATCC) using dye exclusion as well as other primary normal human cells, as described in Task I above.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Demonstrated the sensitivity human prostate cancers to PKC δ inhibition
- Showed activity of PKC δ inhibition against human prostate cancer stem cells
- Designed and synthesized 36 new compounds as PKC δ inhibitors
- Tested the activity of these 36 new compounds against PKC δ and PKC α
- Tested the activity of these 36 new compounds against human prostate cancer cells
- Tested the activity of these 36 new compounds against human prostate cancer *stem* cells
- Established MTD for our lead compound
- Determined the duration of exposure to PKC δ inhibitor drug necessary to achieve maximal cytotoxicity
- Demonstrated that our lead 3rd generation compound (B106) has 5-10 greater potency in inducing cytotoxicity against a panel of human prostate cancer cell than LC-1.
- Demonstrated that our lead 3rd generation compound (B106) has 5-10 greater potency than LC-1 in inducing cytotoxicity against a variety of prostate cancer stem cells.
- Demonstrated that our lead 3rd generation compound (B106) is relatively non-toxic to “normal” human epithelial and primary human endothelial cells in culture.
- Demonstrated that our lead 3rd generation compound (B106) is not toxic when infused directly into the skin of a mouse over 7 days.
- Demonstrated that rapid cell proliferation is not necessary for tumor susceptibility to PKC δ inhibitors.
- Demonstrated that our lead 3rd generation compound (B106) has 5-10 greater potency than LC-1 in inducing cytotoxicity against a variety of prostate cancer stem cells.
- Demonstrated that our lead 3rd generation compound (B106) can be packaged into liposomes without loss of cytotoxic activity.
- Designed a strategy for synthesis of more hydrophilic analogs of our 3rd generation lead compound.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Asami Takashima & Douglas V Faller: Targeting the RAS oncogene. Expert Opin. Ther. Targets (2013) in press.

CONCLUSION:

In our first two years of work, we have made substantial progress. We have succeeded in demonstrating that multiple types of human prostate cancer cells are susceptible to PKC δ inhibition, using siRNA as a “specificity” test, and multiple structurally-distinct small molecule PKC δ inhibitors. These findings validate PKC δ as a target in prostate cancer, and provide proof-of-principle for the use of PKC δ inhibitors as potential therapeutics. Furthermore, we have shown the utility of PKC δ inhibition as a strategy for the elimination of prostate cancer stem cells. We have refined the initial PKC δ inhibitor lead compound now through 2 generations, producing small molecules of increasing potency and PKC δ specificity. Our next generation will be optimized for “drug-like” properties, to facilitate moving into *in vivo* testing of tumor xenografts.

This *in vivo* testing in animal models has not yet proven successful due to the chemical properties of the lead 3rd generation molecule, but two alternative strategies are moving forward. Results of such studies will demonstrate the efficacy of this approach, provide informal toxicology, and informal PK.

REFERENCES:

1. Xia S et al. PKCdelta survival signaling in cells containing an activated p21Ras protein requires PDK1. *Cell Signal*. 21:502-508 (2009).
2. Xia S, Forman LW, and Faller DV. Protein Kinase C{delta} is required for survival of cells expressing activated p21RAS. *J Biol.Chem*. 282:13199-13210 (2007).
3. Chen Z et al. The proliferation and survival of human neuroendocrine tumors is dependent upon protein kinase C-delta. *Endocr.Relat Cancer* 18:759-771 (2011).
4. Iglehart JD and Silver DP. Synthetic lethality--a new direction in cancer-drug development. *N.Engl.J.Med*. 361:189-191 (2009).
5. Kaelin WG, Jr. The concept of synthetic lethality in the context of anticancer therapy. *Nat.Rev.Cancer*. 5:689-698 (2005).
6. Li Y and Littera J. Cancer stem cells: distinct entities or dynamically regulated phenotypes? *Cancer Res*. 72:576-580 (2012).
7. Reya T et al. Stem cells, cancer, and cancer stem cells. *Nature* 414:105-111 (2001).
8. Dick JE. Stem cell concepts renew cancer research. *Blood*. 112:4793-4807 (2008).
9. Clevers H. The cancer stem cell: premises, promises and challenges. *Nat.Med*. 17:313-319 (2011).
10. Nguyen LV et al. Cancer stem cells: an evolving concept. *Nat.Rev.Cancer*. 12:133-143 (2012).
11. Franken NA et al. Clonogenic assay of cells in vitro. *Nat.Protoc*. 1:2315-2319 (2006).
12. Pastrana E, Silva-Vargas V, and Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell*. 8:486-498 (2011).
13. Singh SK et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res*. 63:5821-5828 (2003).
14. Ponti D et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res*. 65:5506-5511 (2005).

15. Ricci-Vitiani L et al. Identification and expansion of human colon-cancer-initiating cells. *Nature*. 445:111-115 (2007).
16. Eramo A et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death.Differ.* 15:504-514 (2008).
17. Han ME et al. Cancer spheres from gastric cancer patients provide an ideal model system for cancer stem cell research. *Cell Mol.Life Sci.* 68:3589-3605 (2011).

APPENDICES: None

SUPPORTING DATA: included above